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13. ABSTRACT ***(Maximum 200 Words)*** Breast cancer metastasizes to bone more frequently than any other tumor but the mechanisms responsible remain unclear. Parathyroid hormone-related protein (PTHrP) is an established tumor product which produces hypercalcemia by stimulating osteoclastic bone resorption. Recently, a role for PTHrP in the establishment of breast cancer metastases in bone has been proposed. PTHrP acts via the common parathyroid hormone/PTHrP receptor (PTHR), which has been demonstrated in breast cancer cell lines and metastatic breast tumors. These findings suggest that an autocrine and/or paracrine loop between PTHrP and its receptor is involved in the establishment of breast cancer metastases in bone. The proposed work will test the hypothesis that an autocrine/paracrine loop between PTHrP and the PTHR is involved in the establishment of breast cancer metastases in bone. Enhancement of this loop, by either increasing receptor expression, constitutive activation of the receptor or by TGFβ stimulation, should increase the metastasis formation. Conversely, interruption of this loop, by overexpression of a signaling-deficient receptor, should reduce metastasis. Breast cancer lines will be produced which stably express wild-type, constitutively active or signaling-deficient PTHR. Cell lines will be characterized in vitro for growth and PTHrP production and in vivo for the development of bone metastases. The first year of this three-year award has resulted in significant training and research accomplishments in 1) laboratory molecular biology techniques as well construction of stable cell lines which will facilitate the study of breast cancer metastases to bone; 2) data analysis and public presentation skills; 3) mechanisms of cancer metastases with respect to bone metastases. My research accomplishments have exceeded the original statement of work in that I have accomplished that proposed for year one in addition to studies which determine the signaling pathways responsible for the TGFβ induction of PTHrP.

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INTRODUCTION

Breast cancer metastasizes to bone more frequently than any other tumor type resulting in the complications of hypercalcemia, pain, fracture and nerve compression syndromes. Althought Paget proposed the "seed and soil" hypothesis to explain this phenomenon over a century ago (Paget 1889), the precise mechanisms by which breast cancer cells cause bone lesions are still unclear. Approximately 70% of women dying from breast cancer have bone metastases and, of these, 10-20% will be hypercalcemic (Coleman and Rubens 1987).

Parathyroid hormone-related protein (PTHrP) is an established tumor product which produces hypercalcemia by stimulating osteoclastic bone resorption and renal tubular calcium reabsorption (Guise and Mundy 1998). PTHrP was demonstrated in 92% of breast cancer metastases to bone compared with 17% of metastases to other sites (Powell et al., 1991), and subsequent in situ hybridization studies showed PTHrP mRNA to be present in 73% of bone metastases and 20% of metastases to other sites (Vargas et al., 1992). These studies strongly imply a role for PTHrP in the establishment of breast cancer metastases in bone, which is supported by the observation that an antibody against the amino-terminus of PTHrP blocked the formation of osteolytic bone lesions and the growth of the metastatic deposits in the bones of nude mice inoculated with a PTHrP-producing breast cancer cell line (Guise et al., 1996).

The amino-terminus of PTHrP acts via the common parathyroid hormone/PTHrP receptor (PTHR), a G protein-coupled, seven transmembrane domain receptor (Jüppner et al., 1991). This receptor has been demonstrated in breast cancer cell lines (Birch et al., 1995) and primary and metastatic breast tumors (Carron et al., 1997; Downey et al., 1997a, 1997b), with expression being greater in metastases when compared with primary tumor (Downey et al., 1997a).

These findings suggest that an autocrine and/or paracrine loop between PTHrP and its receptor is involved in the successful establishment of breast cancer metastases in bone. Additionally, the action of PTHrP in the establishment of bone metastases may be potentiated by growth factors released from bone as a result of osteolysis (Yin et al., 1999). One such factor is transforming growth factor (TGF)-β, a growth factor present in high concentrations in bone matrix (Hauschka et al., 1986) and which is a positive regulator of both PTHrP (Yin et al., 1999) and PTHR expression (Schneider et al., 1992).

The proposed work will test the **hypothesis** that an autocrine/paracrine loop between PTHrP and the PTHR is involved in the successful establishment of breast cancer metastases in bone. Enhancement of this loop, by either increasing receptor expression, constitutive activation of the receptor or by TGFβ stimulation, should increase the initiation and progression of metastasis formation. Conversely, interruption of this loop, by overexpression of a signaling-deficient receptor, should reduce metastasis. To determine the role of PTHR in breast cancer metastases to bone, breast cancer lines will be produced which stably express wild-type, constitutively active or signaling-deficient PTHR. These cell lines will be characterized in vitro for growth and PTHrP production and in vivo for the development of bone metastases.

BODY: Annual Summary of Training and Research Accomplishments to Date

Training

During the past year, this postdoctoral fellowship has provided training in the following laboratory techniques: 1) recombinant DNA technology (preparation of cDNA expression

vectors, PCR, RT-PCR, transfection, construction of stable cell lines), 2) immunoassay techniques (ELISA, RIA, Western blot), 3) determination of receptor numbers by Scatchard analysis (see appended example), 4) in vivo bone metastases assay, 5) bone histomorphometry, 6) statistical and graphical analysis of data. In addition to these technical aspects of the proposed research projects, there has been additional training in 1) data presentation, 2) public speaking, 3) reviewing the literature. Specifically, I present data from this project once per month at the large Endocrinology laboratory meeting in addition to weekly at Dr. Guise's laboratory meeting. The Division of Endocrinology hosted a 2-day seminar for postdoctoral fellows and faculty to enhance public speaking practices. I attended this, and used my newly acquired skills to present a one-hour research seminar for the Division of Endocrinology Research Seminar Series. Finally, I presented the research supported from this postdoctoral fellowship at the European Calcified Tissue Society Meeting in Tampere, Finland (May 2000) and the American Society for Bone and Mineral Research, Toronto, Canada (September 2000). I also attended the San Antonio Breast Cancer Symposium in December, 1999. At these meetings, I learned different aspects of research in the area of breast cancer metastases to bone which were presented by investigators from other universities.

Research Accomplishments

Research accomplishments are described below and categorized into 1) those which fall under the approved Statement of Work and 2) related studies which were not described in the original Statement of Work:

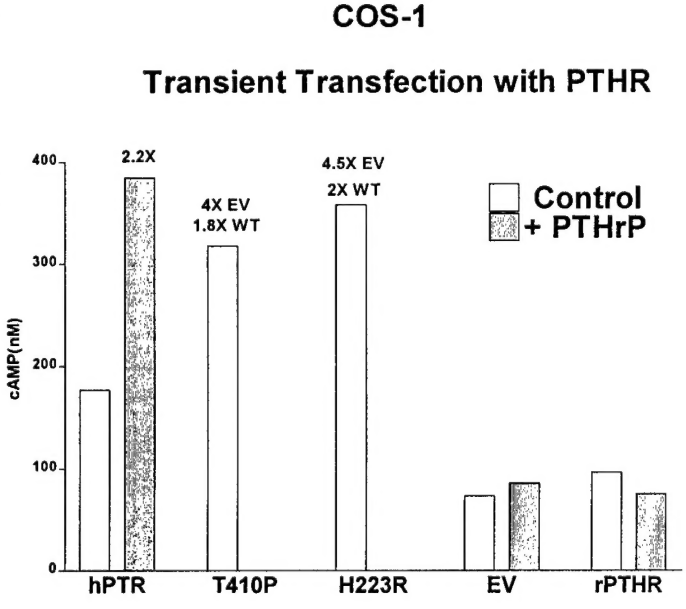
Research Accomplishments From Approved Statement of Work for Year 1

Task 1 (Year 1, Months 1-2): Prepare wild-type, constitutively active and signaling deficient PTHR cDNA expression vectors. The wild-type human PTHR, and two different constitutively active PTHRs (H223R and T410P) cDNAs were subcloned into both the pcDNA3neo^r and pIRES expression vectors. A signaling deficient mutant (R233H/Q451K) was constructed by site-directed mutagenesis of the human wild-type PTHR. This mutation, expressed in rat PTHR, was shown to bind PTHrP, but not signal. Since this area of the rat PTHR was very similar to the human PTHR, we reasoned that a similar mutation in the human receptor would result in PTHrP binding without signal transduction. This PTHR(R233H/Q451K) mutant cDNA was also subcloned into pcDNA3 and pIRES expression vectors. This task took approximately 4 months, as completion of the site directed mutagenesis took longer than expected.

Task 2: (Year 1, Months 2-12): Transfect MDA-MB-231 cells with wild-type and mutant PTHR cDNAs and establish stable overexpressing cell lines. The wild-type human PTHR, two different constitutively active PTHRs (H223R and T410P) and PTHR(R233H/Q451K) signaling deficient mutant cDNAs were transfected into MDA-MB-231 human breast cancer lines using lipofectamine procedure. Prior to stable transfection, the constructs were expressed into COS cells and cAMP accumulation in the presence or absence of PTH was assessed (FIGURE 1). These results demonstrate that cAMP accumulation was increased in response to PTHrP in COS cells transfected with wild-type human PTHR. In contrast, empty vector DNA or rat PTHR expression in COS cells did not result in cAMP accumulation in response to PTHrP. Constitutively active PTHR (H223R) and (T410P) expression in COS cells caused cAMP accumulation in the absence of the ligand PTHrP, consistent with

previously published work (Schipani et al.,1995 and 1996). Since these data indicated that transfection of the respective PTHR cDNAs resulted in cell surface expression of the respective receptors which had the capacity to bind the ligand, PTHrP, the respective cDNAs were transfected into MDA-MB-231 cells. Pools of the stable transfectants were cloned by limiting dilution in the presence of the selective marker, G418, and screened for receptor expression by measuring cAMP accumulation in the presence or absence of PTHrP. Approximately 50 clones were screened for each PTHR cDNA as well as for the empty vector control. FIGURES 2A-C show the results of cAMP accumulation by the positive stable MDA-MB-231 clones which express wild-type or constitutively active PTHR in response to PTHrP (FIGURE 2A) or in the absence of PTHrP (FIGURE 2B & C). Figure 2A shows that only the MDA-MB-231 clones which expressed the constitutively active PTHR(H223R) demonstrated cAMP accumulation in the absence of the PTHrP ligand. The wild-type and both constitutively active PTHR(H223R) and (T410P) had increased cAMP in response to PTHrP as did the positive control, ROS (rat osteoblastic sarcoma) cell line. There were no functional PTHR expressed by the empty vector clones. Figures 2B and 2C represent a time-course of cAMP accumulation in the stable MDA-MB-231 clones in the absence of PTHrP. In this experiment, both MDA-MB-231 clones which expressed PTHR(H223R) (FIGURE 2B) or (T410P) (FIGURE 2C) had cAMP accumulation over 60 minutes. This constitutive activity was greater in the PTHR (H223R) clones and is consistent with previously published results (Schipani et al., 1995 and 1996). The results of Scatchard analysis for receptor numbers in the respective clones are demonstrated in TABLE 1; an example of such an analysis follows. All MDA-MB-231 clones which expressed wild-type or constitutively active PTHR had significant receptor binding, while those that expressed empty vector or the signaling deficient PTHR(R233H/Q451K) had no receptor binding. Thus, it appears that the signaling deficient PTHR(R233H/Q451K) clones do not express the mutant receptors or the receptors do not bind PTHrP.

FIGURE 1: cAMP accumulation in COS cells which have been transiently transfected with wild-type or mutant human PTHR. COS cells which express wild-type PTHR have increased cAMP accumulation in response to PTHrP while those which express empty vector (EV) or rat PTHR (rPTHR) do not. In contrast, there is constitutive cAMP accumulation in those cells which express PTHR (T410P) or (H223R). Values represent the mean of 2 measurements.



cAMP Accumulation in Response to PTHrP
MDA-MB-231 Clones Which Express WT or Mutant PTHR

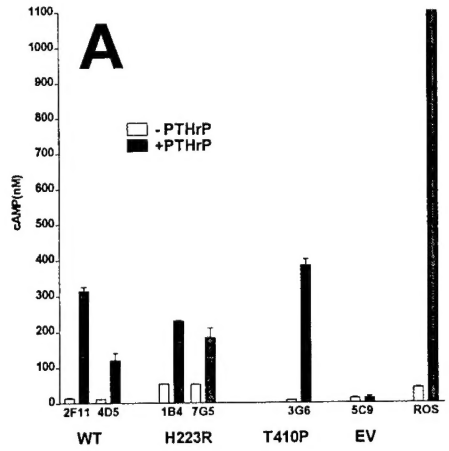
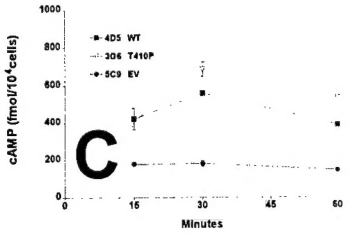


FIGURE 2A: cAMP accumulation in stable MDA-MB-231 clones which express wild-type or mutant human PTHR. All clones except the empty vector clone (EV) have increased cAMP accumulation in response to PTHrP. ROS are rat osteosarcoma cells which serve at the positive control. At this time point (2 hours), only the PTHR(H223R) clones have cAMP accumulation in the absence of ligand (PTHrP) which is greater than the basal state. N=3 measurements per group.

FIGURE 2B & C: Time course of cAMP accumulation in the absence of the ligand PTHrP in stable MDA-MB-231 clones which express wild-type or mutant human PTHR. 2B: Stable clones which express PTHR(H223R) have increase cAMP accumulation over time in the absence of PTHrP. 2C: Stable clones which express wild-type (WT) or T410P PTHRs have slightly more cAMP accumulation in the absence of PTHrP compared with the empty vector clone. N=3 measurements per group.

Time Course of cAMP Accumulation in MDA-MB-231 clones which express WT and Mutant PTHR



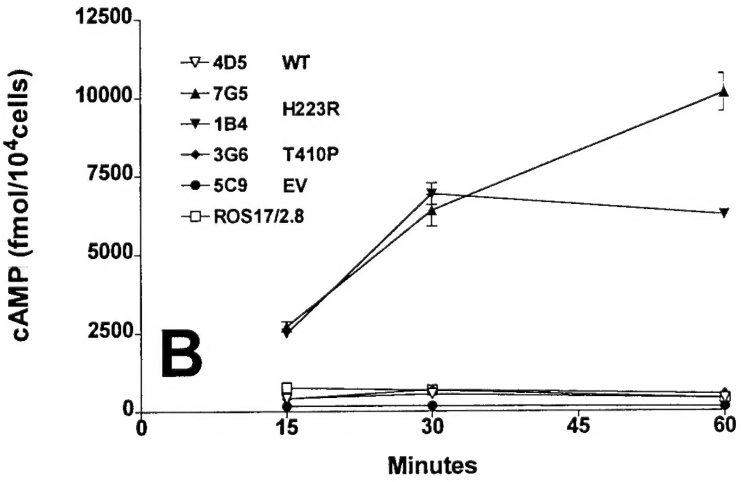


Table 1

Cell line	Mutation	Description	Affinity constant Ka (x10^6 L/mol)	Receptors/cell (x10^7)
5C9	-	empty vector	-	-
2F11	-	wild type	33.3	1.4
4D5	-	wild type	12.5	3.09
1B4	H223R	constitutively active	7.45	6.1
7G5	H223R	constitutively active	14.2	6.55
3G6	T410P	constitutively active	7.15	5.21
1C10	R233H/Q451K	inactive (binds PTHrP, does not signal)		-
3G8	R233H/Q451K	inactive (binds PTHrP, does not signal)	46.4*	0.0395*

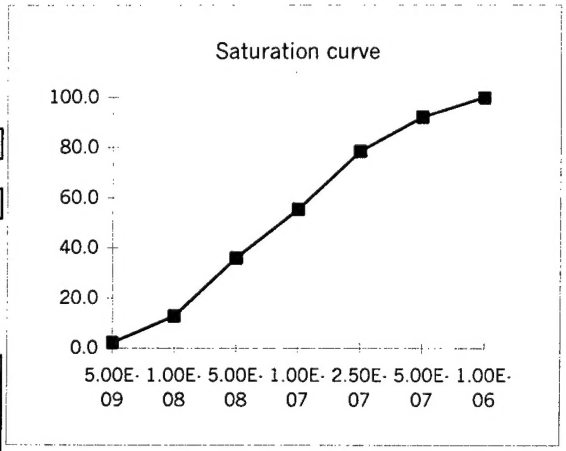
* Low counts, results not reliable.

PTHrP binding to PTH-receptor cell lines

Name	Sanna
Assay	99-12
Date	9/24/99
Cell line	**4D5**

Note:PTHR wild type

Tracer stock concentration (M)	4.54E-08
Tracer concentration (M)	4.54E-11
Amout of cells/ml	98560
SD	15800
volume (µl)	**500**
Total response (cps)	**106828.3**
^{125}I/label	**1**



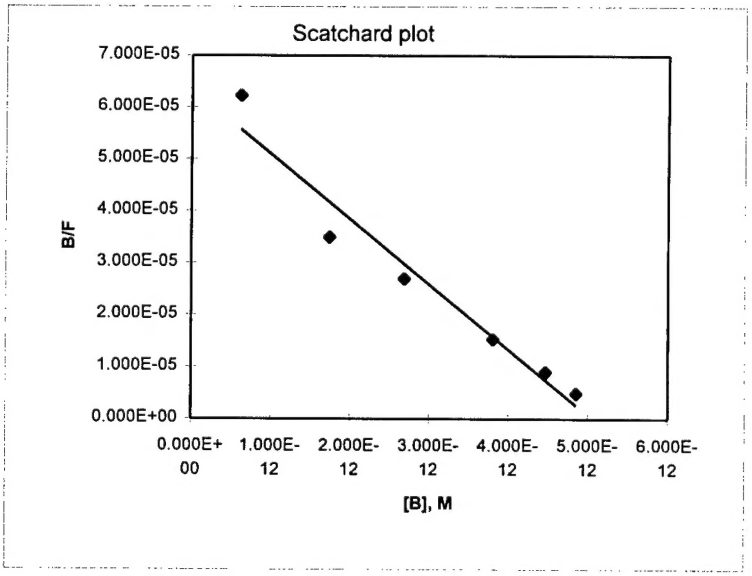
bg corr. cps	*[Cold ligand]*	*B/Bo %*	*B [x]*	*F=T-B [x]*	*B/F*	*fit*
254.2	**5.00E-09**	2.2				
1463.25	**1.00E-08**	12.8	6.219E-13	1.00E-08	6.219E-05	5.568E-05
4097.25	**5.00E-08**	35.9	1.741E-12	5.00E-08	3.483E-05	4.165E-05
6320.85	**1.00E-07**	55.3	2.686E-12	1.00E-07	2.686E-05	2.980E-05
8960.75	**2.50E-07**	78.5	3.808E-12	2.50E-07	1.523E-05	1.573E-05
10518.4	**5.00E-07**	92.1	4.470E-12	5.00E-07	8.940E-06	7.429E-06
11421.3	**1.00E-06**	100.0	4.854E-12	1.00E-06	4.854E-06	2.618E-06

affinity constant Ka (L/mol)	**1.25E+07**
total [x] (M)	**5.06E-12**

y = ax + b

correlelation

a = -1.3.E+07
b = 6.35E-05
r^2 = 0.9987
r= **0.99933**



total [x] (M)	**5.06E-12**
Amount of cells /well	98560
PTHrP M/cell	5.14E-17
Avogadro's constant	6.02E+23
PTHrP binding sites/cell	**3.1E+07**

Summary

Cell line	**4D5**
Amount of receptors/cell	**3.09E+07**
Affinity constant Ka (L/mol)	**1.25E+07**

Related Research Accomplishments Performed in Addition to Approved Statement of Work

In addition to the above data, the regulation of tumor-produced PTHrP by TGFβ was investigated in MDA-MB-231 cells since substantial data support central roles for bone-derived TGFβ and tumor-derived PTHrP in a vicious cycle of local bone destruction in osteolytic metastases. TGFβ, stored in bone matrix and released in active form during osteoclastic resorption, stimulates PTHrP production by tumor cells. PTHrP in turn mediates bone destruction by stimulating osteoclasts. A dominant negative type II TGFβ receptor, stably expressed in MDA-MB-231 breast cancer line, inhibited TGFβ-induced PTHrP secretion and development of bone metastases. However, the signaling pathways by which TGFβ increases PTHrP secretion by tumor cells are unknown. In many cell types, TGFβ mediates its effects via cell-surface serine/threonine kinase receptors to the intracellular mediators known as Smads, but there is also evidence for other signaling pathways. To determine the role of the Smad proteins, we stably expressed wild type and dominant-negative mutants of Smads 2, 3, and 4 into MDA-MB-231 cells. The lines were characterized for PTHrP production in response to TGFβ and for signaling by transient transfection with the TGFβ-responsive 3TP-Lux reporter and luciferase assays. Compared to parental cells and empty vector controls, overexpression of wt Smads 2 and 4 enhanced both PTHrP production and 3TP-Lux luciferase activity in response to TGFβ while the dominant negative TGFβ receptor-expressing cells were unresponsive to TGFβ in both assays. In contrast, dominant-negative Smads [Smad2(3S-A), Smad3(3S-A), Smad3(D407E), Smad4(1-514)] reduced, but failed to suppress, TGFβ-stimulated PTHrP secretion. The results suggested both Smad-dependent and -independent TGFβ signaling pathways in breast cancer cells. To examine Smad-independent TGFβ signaling, MDA-MB-231 cells were treated with inhibitors of MAP kinase pathways as well as protein kinases C, A and G, and PI3 kinase. Two specific inhibitors of the p38 MAP kinase pathway significantly reduced both basal and TGFβ-stimulated PTHrP production in parental MDA-MB-231 cells. In the presence of either p38 inhibitor, TGFβ-stimulated PTHrP production was further reduced in MDA-MB-231 clones which expressed any of the dominant-negative Smads. The combination of Smad2 or Smad3 dominant-negative blockade and p38 MAP kinase inhibition resulted in greater inhibition of TGFβ-stimulated PTHrP production than either modality alone. An Erk pathway-specific inhibitor of MAP kinase kinase significantly reduced basal PTHrP production by parental MDA-MB-231 cells as well as basal and TGFβ-stimulated PTHrP production in those that expressed dominant-negative Smads. Inhibitors of protein kinases C, A or G, as well as PI3 kinase had no effect on PTHrP production. In sum these data support both Smad-dependent and -independent pathways for the TGFβ stimulation of PTHrP production by breast cancer cells. The p38, Erk and JNK MAP kinase pathways appear to be a major component of this Smad-independent signaling by TGFβ and represent new molecular targets for anti-osteolytic therapy.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of stable MDA-MB-231 breast cancer cell lines which express wild-type or constitutively active PTHR mutants.
- Determination that the TGFβ regulation of PTHrP production by MDA-MB-231 cells is via both Smad and MAP kinase pathways.

REPORTABLE OUTCOMES

Presentations

S-M Käkönen, JM Chirgwin, K Selander, BG Grubbs, JJ Yin and TA Guise. TGFβ Stimulates Tumor Production of PTHrP via Smad and MAP Kinase Signaling Pathways. American Society for Bone and Mineral Research. September 2000, Toronto, Canada.

S-M Käkönen, JM Chirgwin, K Selander, BG Grubbs, JJ Yin, and TA Guise. Transforming growth factor β (TGFβ) stimulates tumor production of parathyroid hormone-related protein (PTHrP) via Smad-dependent and –independent mechanism. 27th European Symposium on Calcified Tissues, Tampere, Finland, May 2000.

Patents: none

Degrees obtained: none

Development of cell lines: MDA-MB-231 clones which express wild-type or mutant PTHRs as well as those which express wild-type or dominant-negative Smads.

Informatics: none

Funding opportunities based on work supported by this award:

1. Academy of Finland, Grant for researcher training and research done abroad, "Tumor cell-bone interactions: Molecular targets for therapeutic intervention in breast cancer metastases to the skeleton". Awarded for period 6/2000-5/2001, 60,000 FIM ($9,500).
2. Finnish Cultural Foundation, Personal research grant for postdoctoral training abroad, "Tumor cell-bone interactions: Molecular targets for therapeutic intervention in breast cancer metastases to the skeleton". Awarded for period 6/2000-5/2001, 60,000 FIM ($9,500).
3. Maud Kuistila Foundation, Personal research grant for postdoctoral training abroad, "Tumor cell-bone interactions: Molecular targets for therapeutic intervention in breast cancer metastases to the skeleton". Awarded for period 6/2000-5/2001, 60,000 FIM ($9,500).

Employment or Research Opportunities applied for based on work supported by this award: none

CONCLUSIONS

The first year of this three-year award has resulted in significant training and research accomplishments. I have received training in 1) laboratory molecular biology techniques as well construction of stable cell lines which will facilitate the study of breast cancer metastases to bone; 2) data analysis and public presentation skills; 3) mechanisms of cancer metastases with respect to bone metastases. My research accomplishments have exceeded the original statement of work in that I have accomplished that proposed for year one in addition to studies which determine the signaling pathways responsible for the TGFβ induction of PTHrP. Finally, I have had the opportunity during this first year to present my research findings at the national and international level. This postdoctoral fellowship has fueled my research career in breast cancer.

Plans for year 2 include completion of Tasks 4-6 of the Statement of Work:

Task 4: Measure PTHrP production by stable overexpressing cell lines generated in year 1 in the presence and absence of exogenous TGFβ.
Task 5: Determine the adherent and anchorage independent growth characteristics of stable overexpressing cell lines from year 1 in the presence and absence of exogenous PTHrP.
Task 6: Determine in vitro invasive capacity of stable overexpressing cell lines from year 1 in the presence and absence of exogenous PTHrP and TGFβ.

In addition to the above plans, I will begin to learn the in vivo models of bone metastases in preparation for the planned in vivo studies during year 3. Furthermore, I will continue to study the mechanisms by which TGFβ stimulates PTHrP production by tumor cells.

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APPENDIX

The following abstracts are appended:

S-M Käkönen, JM Chirgwin, K Selander, BG Grubbs, JJ Yin and TA Guise. TGFβ Stimulates Tumor Production of PTHrP via Smad and MAP Kinase Signaling Pathways. American Society for Bone and Mineral Research. September 2000, Toronto, Canada.

S-M Käkönen, JM Chirgwin, K Selander, BG Grubbs, JJ Yin, and TA Guise. Transforming growth factor β (TGFβ) stimulates tumor production of parathyroid hormone-related protein (PTHrP) via Smad-dependent and –independent mechanism. 27th European Symposium on Calcified Tissues, Tampere, Finland, May 2000.

Transforming Growth Factor β (TGFβ) Stimulates Tumor Production of Parathyroid Hormone-related Protein (PTHrP) via Smad-dependent and -independent Mechanisms. S-M Käkönen, JM Chirgwin, K Selander, BG Grubbs, JJ Yin and TA Guise. University of Texas Health Science Center at San Antonio

TGFβ, stored in bone matrix and released in active form during osteoclastic resorption, stimulates PTHrP production by tumor cells. PTHrP in turn mediates bone destruction by stimulating osteoclasts. Substantial data support central roles for bone-derived TGFβ and tumor-derived PTHrP in a vicious cycle of local bone destruction in osteolytic metastases. In particular, a dominant negative mutant of the type II TGFβ receptor when stably transfected into the MDA-MB-231 breast cancer cell line, inhibited TGFβ-induced PTHrP secretion *in vitro* and development of bone metastases in a mouse model. However, the signaling pathways by which TGFβ increases PTHrP secretion from tumor cells are unknown. To test whether the major effect is via the Smad proteins, we stably expressed a variety of wild type and dominant-negative mutants of Smads 2, 3, and 4 in the MDA-MB-231 cell line. The lines were characterized for PTHrP production in response to TGFβ and for signaling by transient transfection with the TGFβ-responsive 3TP-Lux reporter and luciferase assays. Compared to parental cells and empty vector controls, the dominant negative TGFβ receptor-expressing cells were unresponsive to TGFβ in both assays. In contrast, a series of dominant-negative Smads [Smad2(3S-A), Smad3(3S-A), Smad3(D407E), Smad4(1-514)] failed to suppress TGFβ stimulated PTHrP secretion by MDA-MB-231 cells. These results support a Smad-independent TGFβ signaling pathway in breast cancer cells. On the other hand, overexpression of wt Smads 2 and 4 enhanced both PTHrP production and 3TP-Lux luciferase activity in response to TGFβ. In sum the data support both Smad-dependent and -independent pathways for the TGFβ stimulation of PTHrP production by breast cancer cells capable of causing osteolytic metastases.

TGFβ Stimulates Tumor Production of PTHrP via Smad and MAP Kinase Signaling Pathways. S-M Käkönen, JM Chirgwin, K Selander, BG Grubbs, JJ Yin and TA Guise. University of Texas Health Science Center at San Antonio

Substantial data support central roles for bone-derived TGFβ and tumor-derived PTHrP in a vicious cycle of local bone destruction in osteolytic metastases. TGFβ, stored in bone matrix and released in active form during osteoclastic resorption, stimulates PTHrP production by tumor cells. PTHrP in turn mediates bone destruction by stimulating osteoclasts. A dominant negative type II TGFβ receptor, stably expressed in MDA-MB-231 breast cancer line, inhibited TGFβ-induced PTHrP secretion and development of bone metastases. However, the signaling pathways by which TGFβ increases PTHrP secretion by tumor cells are unknown. In many cell types, TGFβ mediates its effects via cell-surface serine/threonine kinase receptors to the intracellular mediators known as Smads, but there is also evidence for other signaling pathways. To determine the role of the Smad proteins, we stably expressed wild type and dominant-negative mutants of Smads 2, 3, and 4 into MDA-MB-231 cells. The lines were characterized for PTHrP production in response to TGFβ and for signaling by transient transfection with the TGFβ-responsive 3TP-Lux reporter and luciferase assays. Compared to parental cells and empty vector controls, overexpression of wt Smads 2 and 4 enhanced both PTHrP production and 3TP-Lux luciferase activity in response to TGFβ while the dominant negative TGFβ receptor-expressing cells were unresponsive to TGFβ in both assays. In contrast, dominant-negative Smads [Smad2(3S-A), Smad3(3S-A), Smad3(D407E), Smad4(1-514)] reduced, but failed to suppress, TGFβ-stimulated PTHrP secretion. The results suggested both Smad-dependent and -independent TGFβ signaling pathways in breast cancer cells. To examine Smad-independent TGFβ signaling, MDA-MB-231 cells were treated with inhibitors of MAP kinase pathways as well as protein kinases C, A and G, and PI3 kinase. Two specific inhibitors of the p38 MAP kinase pathway significantly reduced both basal and TGFβ-stimulated PTHrP production in parental MDA-MB-231 cells. In the presence of either p38 inhibitor, TGFβ-stimulated PTHrP production was further reduced in MDA-MB-231 clones which expressed any of the dominant-negative Smads. The combination of Smad2 or Smad3 dominant-negative blockade and p38 MAP kinase inhibition resulted in greater inhibition of TGFβ-stimulated PTHrP production than either modality alone. An Erk pathway-specific inhibitor of MAP kinase kinase significantly reduced basal PTHrP production by parental MDA-MB-231 cells as well as basal and TGFβ-stimulated PTHrP production in those that expressed dominant-negative Smads. Inhibitors of protein kinases C, A or G, as well as PI3 kinase had no effect on PTHrP production. In sum these data support both Smad-dependent and -independent pathways for the TGFβ stimulation of PTHrP production by breast cancer cells. The p38, Erk and JNK MAP kinase pathways appear to be a major component of this Smad-independent signaling by TGFβ and represent new molecular targets for anti-osteolytic therapy.